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13. ABSTRACT *(Maximum 200 Words)* Angiogenesis, the formation of new capillaries from pre-existing vessels, is essential for tumor progression. This tumor endothelium is derived from normal host tissue and is genetically stable. One tumor vessel may supply as many as 10^6 tumor cells. Angiostatin, a proteolytic fragment of plasminogen, inhibits angiogenesis and thereby growth of primary and metastatic tumors. Radiotherapy is important in the treatment of human cancers, but is often unsuccessful due to tumor cell radioresistance. We are investigating the effects of combined treatment of angiostatin and ionizing radiation (IR) in human prostate cancer xenografts. We increase in tumor regression and delayed regrowth in PC-3 prostate cancer cell xenografts. We also show that effect of this combined treatment is directed to the endothelial cells and not to the PC-3 cancer cells. As a beginning to understanding the mechanism of this interaction, we investigated whether the effect of angiostatin, IR, and angiostatin+IR in vitro. We conclude, therefore, that the interaction of angiostatin and IR is not mediated by apoptosis.

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FOREWORD

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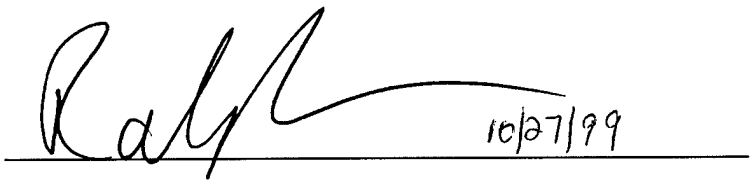

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Introduction

Angiogenesis, the formation of new capillaries from pre-existing vessels, is essential for tumor progression[1-5]. This tumor endothelium is derived from normal host tissue and is genetically stable. One tumor vessel may supply as many as 10^6 tumor cells. Angiostatin, a proteolytic fragment of plasminogen[6], inhibits angiogenesis and thereby growth of primary[7] and metastatic tumors[8-10]. Radiotherapy is important in the treatment of human cancers, but is often unsuccessful due to tumor cell radioresistance[11, 12]. We are investigating the effects of combined treatment of angiostatin and ionizing radiation (IR) in human prostate cancer xenografts. We demonstrate that the combined treatment with angiostatin and IR produces a significant increase in tumor regression and delayed regrowth in PC-3 prostate cancer cell xenografts. We also show that effect of this combined treatment is directed to the endothelial cells and not to the PC-3 cancer cells. As a beginning to understanding the mechanism of this interaction, we investigated whether the effect of angiostatin, IR, and angiostatin+IR was mediated by apoptosis. Our data demonstrate that the percentage of apoptotic endothelial cells does not increase when treated with angiostatin, IR, and angiostatin+IR in vitro. We conclude, therefore, that the interaction of angiostatin and IR is not mediated by apoptosis.

Body

For the first twelve months of the proposal we proposed to use purified angiostatin and ionizing radiation (IR) to assess the anti-tumor effect of combined treatment in prostate cancer. PC-3 cells ($2x10^7$ in 100 µl PBS) were injected into the flank of nude mice and tumors grown to a mean size of 770 mm^3. The animals were divided into four treatment groups: control (n=8), IR (n=10), angiostatin (n=10), combined angiostatin + IR (n=10). Angiostatin was injected intraperitoneally (IP) at a dose of 25 mg/kg/day. IR was delivered over two weeks at a fraction of 500 cGy per day to a total dose of 4000 cGy. Animals receiving combined treatment were injected with angiostatin four hours prior to IR. The tumors were measured twice weekly with calipers. Figure 1 demonstrates the results of this experiment (mean volume ± SEM). The animals treated with angiostatin alone grew at the same rate as the controls. The animals in the control and angiostatin treated groups were euthanized at day 21 when the tumor burden became

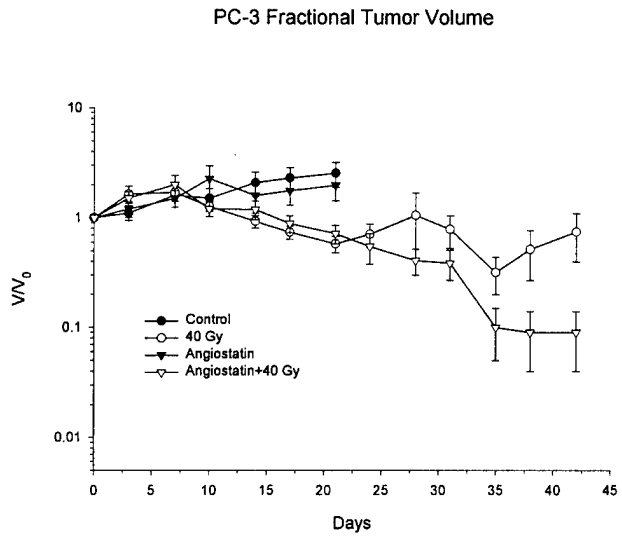


Figure 1

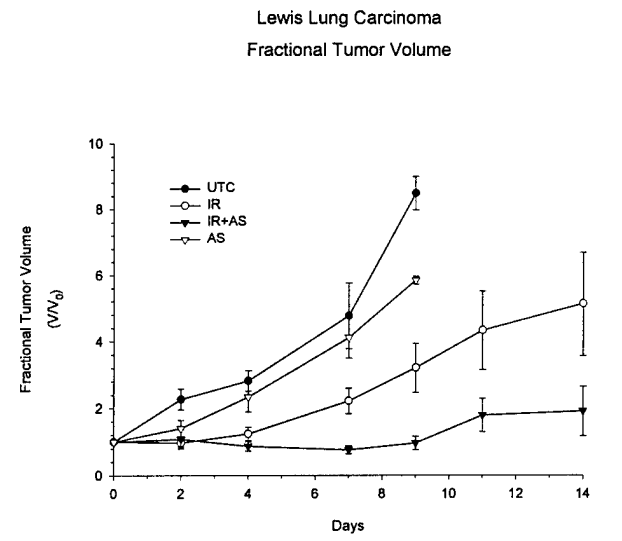


Figure 2

excessive. The animals treated with IR and angiostatin+IR showed similar tumor regression through day 24. From day 24 to day 42 the IR treated tumors began regrowth while the angiostatin+IR treated tumors continued regressing. At day 42 the difference in the means is statistically significant ($P<0.001$).

We also used the Lewis lung carcinoma (LLC) model to study the interaction of angiostatin and IR. LLC cells were injected ($5x10^5$) into C57BL/6 mice. The tumors grew to a mean volume of 1104 mm^3 in 17 days. Twenty-eight mice were divided into four groups of seven. The treatment groups were: untreated control, 40 Gy, angiostatin, and angiostatin + 40 Gy. The 40 Gy exposure was divided into two 20 Gy fractions delivered on day 0 and day 1 of the experiment. Angiostatin was injected IP beginning at day 0 at a dose of 25 mg/kg/day divided into two daily injections and continued for fourteen days. Figure 2 illustrates the results of this experiment. The untreated control animals and the animals treated with angiostatin alone were euthanized on day 9 when the tumor burden became excessive. The time to tumor regrowth is extended in the angiostatin + IR treatment group compared to the IR alone. At day nine the difference in the mean tumor volumes is statistically significant ($P<0.05$). To determine the effects of treatment on tumor neovascularization, representative tissue sections from LLC tumors were stained using anti-CD-31 antibody and standard immunohistochemical techniques. The number of tumor vessels per high power field was reduced following to treatment with angiostatin+IR compared with all other treatments. These data are indicative of endothelial cells being the target of angiostatin+IR.

To demonstrate that the PC-3 tumor cells are not the target of the angiostatin/IR interaction, PC-3 cells were treated with angiostatin and IR *in vitro* employing the clonogenic assay. Figure 3 shows the results of these experiments (mean $\pm$ SEM of three experiments). As can be seen the cells treated with angiostatin have identical surviving fractions to those cells treated with IR alone. This demonstrates that the *in vivo* interaction of angiostatin and IR is not attributable to the direct killing of the PC-3 cells.

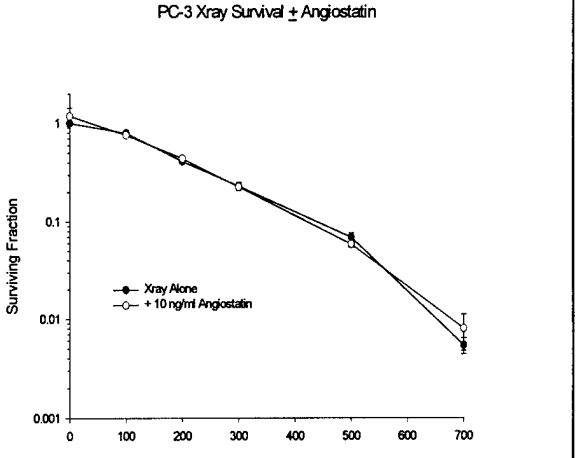


Figure 3

My laboratory has demonstrated that the angiostatin/IR response is directed at the endothelial cells. Figure 4 shows the dose response for human umbilical vein endothelial cells (HUVEC) when treated with angiostatin (1-1000 ng/ml) *in vitro* (mean $\pm$ SEM of three experiments). There is an initial rapid decrease in surviving fraction which levels off at 100 ng/ml and remains comparable when HUVEC are treated with concentrations of angiostatin as high as 1000 ng/ml. In order to ascertain the effects of treatment with angiostatin followed by IR, we utilized the clonogenic assay and HUVEC. In order to conserve the limited angiostatin supply, we chose to use

an angiostatin concentration of 100 ng/ml (since the surviving fraction was the same as 1000 ng/ml) added to the cultures 18 hours after plating. IR was delivered four hours after addition of angiostatin. Figure 5 shows the results (mean ± SEM of three experiments) of these experiments demonstrating the additive nature of the interaction of angiostatin and IR in HUVEC. In order to elucidate whether the mechanism of this killing by angiostatin was due in part to increased apoptosis in angiostatin treated cells, we determined the percentage of apoptotic cells using HUVEC after treatment with 1000 ng/ml angiostatin, 10Gy, or the combination when compared to controls. We chose to use the 7-AAD staining method as it stained both early and late apoptotic cells. HUVEC were treated with angiostatin, IR, or the combination while attached to a tissue culture dish. At the appropriate times (0, 12, 24, and 48 hours), the cells were disaggregated with trypsin/EDTA, washed, and incubated with 7-AAD (20 μg/ml) for thirty minutes. The samples were then analyzed on a Beckman FACS machine. Figure 6 illustrates the results of these experiments. There is no increase in apoptosis in HUVEC treated with angiostatin when compared with control. Also, the cells treated with angiostatin prior to 10 Gy had the same percentage of apoptotic cells as the cells treated with 10 Gy alone. This leads us to conclude that angiostatin does not kill cells through apoptosis, and that the interaction of angiostatin and IR is not mediated by apoptosis.

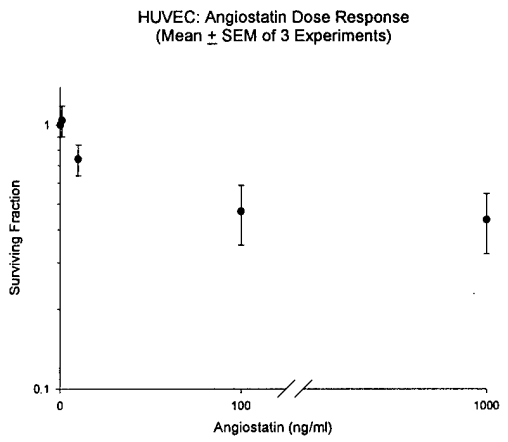


Figure 4

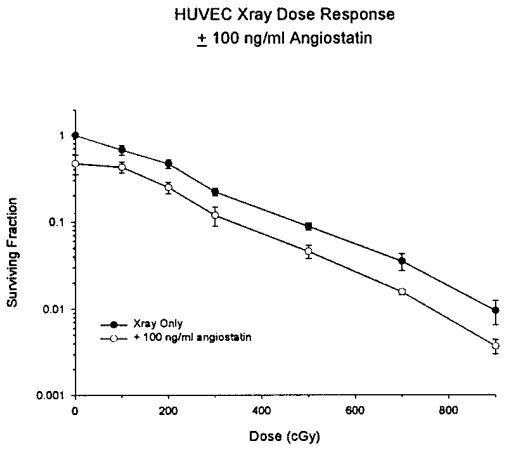
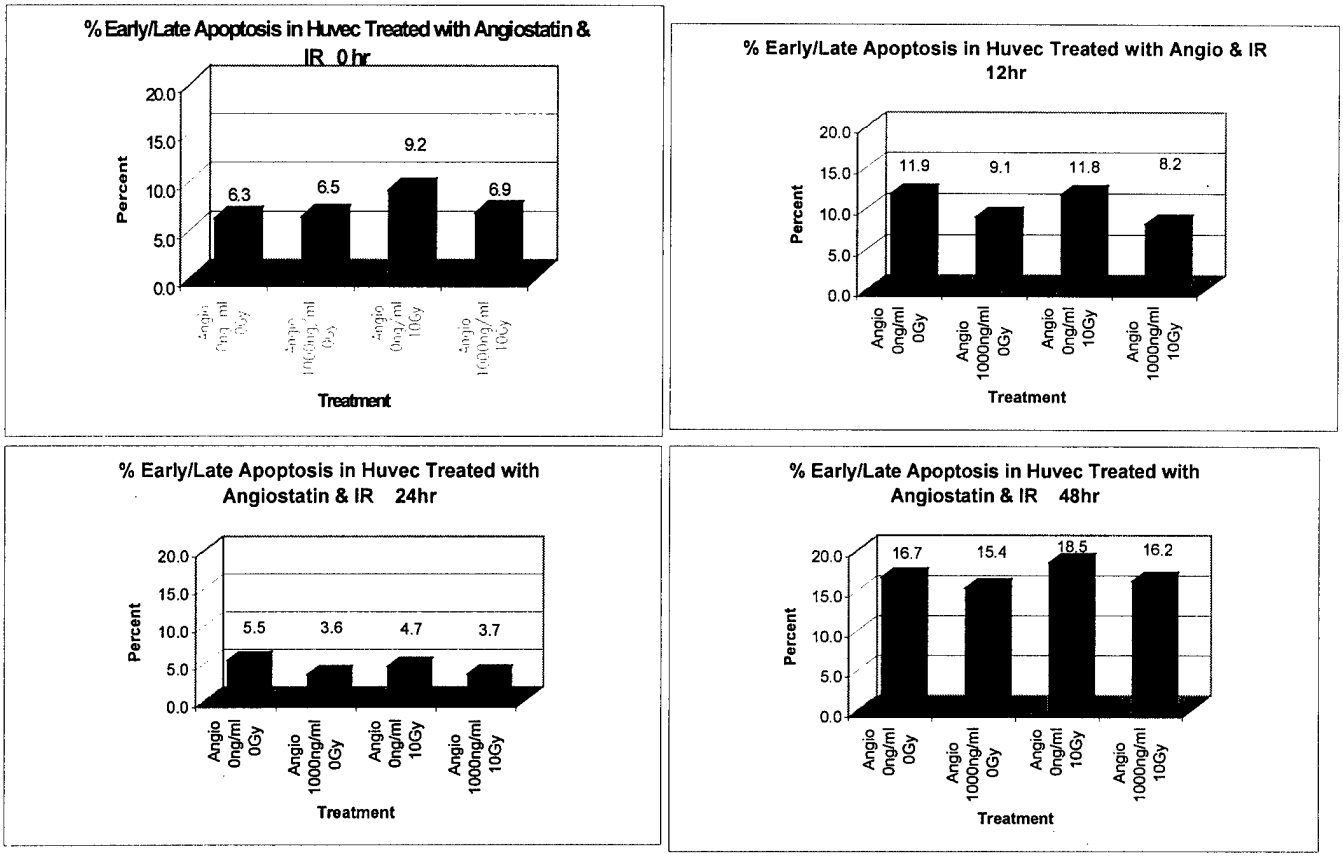


Figure 5

Figure 6


% Early/Late Apoptosis in Huvec Treated with Angiostatin & IR 0 hr
Percent
20.0
15.0
10.0
5.0
0.0
6.3
6.5
9.2
6.9
Treatment
% Early/Late Apoptosis in Huvec Treated with Angio & IR 12hr
11.9
9.1
11.8
8.2
Angio 0ng/ml 0Gy
Angio 1000ng/ml 0Gy
Angio 0ng/ml 10Gy
Angio 1000ng/ml 10Gy
% Early/Late Apoptosis in Huvec Treated with Angiostatin & IR 24hr
5.5
3.6
4.7
3.7
% Early/Late Apoptosis in Huvec Treated with Angiostatin & IR 48hr
16.7
15.4
18.5
16.2

Key Research Accomplishments

- PC-3 prostate cancer cell xenografts treated with angiostatin + IR produce a significant increase in tumor regression and delayed tumor regrowth than xenografts treated with angiostatin or IR alone.
- The effects of treatment with angiostatin + IR is directed to the tumor endothelium and not the PC-3 cancer cells
- The interaction of angiostatin + IR is additive.
- The interaction of angiostatin + IR is not mediated by apoptosis.

Reportable Outcomes

1. A manuscript reporting results of effect of treatment with angiostatin and IR and the mechanism underlying this interaction, specifically pertaining to prostate cancer is in preparation.

Conclusions

We demonstrate that the combined treatment with angiostatin + IR produces a significant increase in tumor regression and delayed regrowth in PC-3 prostate cancer cell xenografts. We also show that effect of this combined treatment is directed to the endothelial cells and not to the PC-3 cancer cells. Our data demonstrate that the percentage of apoptotic endothelial cells does not increase when treated with angiostatin, IR, and angiostatin+IR *in vitro*. We conclude, therefore, that the interaction of angiostatin and IR is not mediated by apoptosis. We continue to investigate the mechanism by which the angiostatin/IR interaction is mediated in order to develop new therapies based on this mechanism.

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Appendices

None